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Original Research Paper

The Potential Impact of Suborbital Flight on Astaxanthin Production and Germination of Cyst Cells in *Haematococcus Pluvialis* Alga

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ABSTRACT

The microalga *Haematococcus pluvialis* is a natural source of astaxanthin, a red carotenoid that accumulates in the cytoplasm of algal cells under stress conditions and is produced from the transition of motile vegetative cells to non-motile cysts. In industrial astaxanthin production, cyst walls are mechanically disrupted to release astaxanthin, which results in loss of cell viability. In this study, the impact of suborbital flight on *H. pluvialis* cells was investigated at two growth stages of aplanospore and cyst for the first time. For flight experiments, samples were placed in the bio-capsule during suborbital flight, and then examined in terms of cyst germination, zoospore release, and astaxanthin production after flight. Results showed that 71% of the cyst cells germinated and released zoospores after 2 days post-flight, and the aplanospore cells tended to accumulate pigments. Cell biomass did not change significantly after 7 days of flight. Spaceflight conditions led to a 2.1 and 1.3-fold rise in H_2O_2 content after 2 and 7 days of flight, respectively. The highest content of astaxanthin (26.1 mg^{-1} g FW) and phenolic compounds (935.6 mg^{-1} g FW) was observed in the samples after 7 days of flight. These findings indicate that *H. pluvialis* can survive spaceflight conditions and may be a promising candidate for inclusion in human life support systems in space.

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NOMENCLATURE

BBM	Bold Basal Medium
GC	Germinated cyst
NM	Non-Motile
M	Motile
ROS	Reactive Oxygen Species

1. INTRODUCTION

Long-term human space missions require a controlled life support system that can recover water, oxygen, and waste cycle [1]. Living on Mars is difficult due to its high dependence on numerous terrestrial facilities and economic investment to send resources. Therefore, a future colonization of Mars should be self-sufficient in terms of air, water, and food production. This goal can be achieved by utilizing the resources available on Mars and the biological waste generated by astronauts. The Mars atmosphere is rich in carbon dioxide, which can be converted into beneficial organic compounds by photosynthetic microorganisms such as cyanobacteria, microalgae or plants [2-5]. In addition, these organisms can grow using compounds from locally available resources such as astronaut urine and Martian regolith to produce edible biomass and nutritional supplements for team members [6,7]. Therefore, studying algae in space conditions could be crucial in designing a regenerative life support system, cloning algae on the Martian surface, and preparing the environment for human life [4]. Algae can be considered a candidate for human life support due to their highly edible biomass, easy transport, and rapid growth. Since the beginning of spaceflight experiments, algae have been intensively studied for this purpose, even before the first human flight into space [8]. *Chlorella* microalga was studied as the first regenerative life support system in the late 1960s to absorb carbon dioxide and release oxygen [2]. Subsequently, numerous experiments investigated the effects of radiation, microgravity, vacuum, and extreme temperature changes on algal growth. The results exhibited that the growth response of algae depends on the alga strain and genetics [5, 6].

Haematococcus pluvialis is a unicellular, green, freshwater microalga widely used in food and pharmaceutical industries. It is a rich source of astaxanthin (comprising about 2-3% of its dry weight) in the cyst stage. Astaxanthin is a potent antioxidant with anti-inflammatory, anti-tumor, anti-UV, and anti-cardiovascular properties. Algal cells have a complex life cycle that is divided into four stages, including the green spindle cells (motile), green aplanospore cells (non-motile), carotenoid-containing aplanospore cells, and red cysts with high levels of astaxanthin accumulation. In the spindle-shaped stage, cells are capable of division and are green due to the presence of the chlorophyll pigment. Under stress conditions, algal cells transform from green motile cells into non-motile aplanospore cells and then red cysts [7, 8]. Upon germination, red cysts release red motile zoospores. In favorable conditions, the red motile zoospores are released from cysts and turn green again, and then the algal life cycle is repeated. The cysts remain dormant for long periods under stress conditions [8, 9]. Cysts contain a resistant wall with a three-layered sheath, a secondary wall, and a tertiary wall. Releasing astaxanthin from cysts is difficult, and the wall should be broken to remove the pigments. In industries, supercritical carbon dioxide is used to release astaxanthin. In research laboratories, homogenizers or ultrasonic devices are also used to break the cyst cells; However, these methods lead to the destruction of zoospores, and cells are died [10]. It is very valuable to achieve a technique that breaks the cyst wall, accompanied by the release of pigments and zoospores.

Microalgae play a crucial role in bioregenerative life support systems, enabling the sustainable maintenance of human life in closed or space environments. Their biological properties make them extremely valuable for maintaining ecological balance and supporting human life. The microalga *H. pluvialis* can be a promising candidate for cultivation in a closed life support system due to its ability to perform a CO_2-O_2 cycle, recycle waste, and produce biomass. Additionally, it has ability to mitigate the effects of space conditions on astronauts during long-duration missions. The fresh astaxanthin can help astronauts mitigate the effects of space radiation on their bodies, protect their eyes, prevent cardiovascular disease, and maintain bone health. The algae biomass can also be used in astronaut food and contains vitamins, minerals, polysaccharides, fiber, amino acids, and essential fatty acids to increase

the astronaut's immune system against microorganisms in space [11, 12].

Sounding rockets are suborbital space vehicles used to carry scientific payloads for short-duration microgravity. The results of suborbital experiments can provide a cost-effective and flexible platform for biological research relevant to designing bioregenerative life support systems (BLSS) [12]. During a suborbital flight, environmental parameters such as temperature, pressure, and gravity vary significantly throughout the mission. However, when the biological samples are enclosed within the bio-capsule, the temperature, vibration, and radiations are carefully controlled at the specific levels, and the samples are exposed to variations in gravity and low pressure. Experiments conducted using sounding rockets demonstrated that short durations of microgravity can lead to metabolic changes, including a temporary increase in the ATP/ADP ratio and alterations in the fructose-2,6-bisphosphate pool [13]. To date, many experiments have been conducted using green algae, cyanobacteria (blue-green algae), or euglenophytes (unicellular microalgae) during launch missions and subsequently in ground-based laboratories by NASA [12, 15]. The results showed that algae employ complex mechanisms to resist environmental stresses. Understanding how these cells respond can contribute to the development of a sustainable life support system. Numerous studies have identified the effects of environmental stress on astaxanthin production in the alga *H. pluvialis*, but there are limited studies on the cultivation of *this alga* under real space flight. Casula et al. (2024) investigated the potential for cultivating *H. pluvialis* in a growth medium derived from Mars regolith and astronaut urine under laboratory conditions. The results showed that *H. pluvialis* could be grown using only these resources, thus theoretically reducing the payload related to fertilizers brought from Earth to zero [12]. The effect of space conditions on the growth of microalga *Anabaena siamensis* was investigated during a satellite flight for 15 days. The results showed that microalgal growth was slower than the control on the ground; however, after several generations, its growth reached the control cells. The data showed that algae can adapt to space environments [15]. Until now, no study has been conducted on the effects of suborbital flight on the cell growth and cyst germination in *H. pluvialis*; this is the first study. Therefore, this study examined the impact of suborbital flight on cell morphology, H_2O_2 level, and the production of valuable metabolites after flight.

2. MATERIALS and METHODS

2.1. Microalga growth conditions

The microalga *Haematococcus pluvialis* (IBRC-M 50096) was purchased from the Iranian biological resource center (IBRC) and cultured in BBM liquid medium. The algae were cultivated in 100 mL Erlenmeyer flasks containing 30 mL of culture medium and an initial cell density of 0.5 × 10 ^5 cells. The cultures were maintained at 25 ± 2°C under white LED light with an intensity of 25 µmol photons m−2 s−1 and agitated on a shaker. After 24 days of cultivation (at the late logarithmic phase), the light intensity was changed to 75 µmol photons m^{-2} s^{-1} for cyst induction [10].

2.2. Preparation of Flight Samples

The algal suspension (2 mL) was uniformly cultured on the solid BBM medium containing low-melting-point agarose (1% (w/v)) in 8 cm diameter Petri dishes. Each Petri dish contained 30 mL of culture medium. The cultures were then put in a growth chamber. The cells were prepared in the green vegetative phase (on day 20 of culture) and the red phase (on day 30), with three replicates for each flight sample (Fig. 1). The control samples were also placed in the flight site in the same conditions.

The suborbital flight was conducted by a sounding rocket on December 5, 2024. Flight samples were loaded into Kavous bio-capsule, which was designed for biological payloads within a BioRok. The launch reached an altitude of 130 kilometers above the Earth's surface. During the flight, the samples experienced microgravity for 230 seconds (about 4 minutes), and the temperature inside the capsule was maintained at 27°C. After recovery, the biological samples were transported to the laboratory. Microscopic examination of algal cells was conducted 48 hours after flight on green and red cyst-containing Petri dishes. The cell growth and biochemical analyses were performed

on the green aplanospore Petri dishes after 2 and 7 days post-flight.

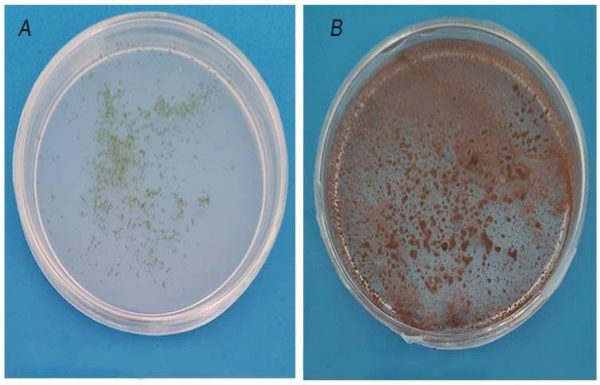

Fig. 1. Depiction of *Haematococcus pluvialis* cells in the green aplanospore (A) and red cyst (B) stages.

2.3. Algal growth kinetics

After culturing the algal cells on solid medium, samples were harvested every 5 days over 30 days. The culture medium containing algal cells was heated at 60°C, and then centrifuged at 5000 × g for 5 minutes. The cell pellets were rinsed twice with distilled water, and their dry weight was evaluated after drying in an oven at 60°C for 24 hours [16].

2.4. Microscopic studies

The algal cells of the flighted and control samples after 2 days of flight were harvested with a sterile loop and added to vials containing 100 µL of distilled water. The cells were studied with a light microscope (Nikon, ECLIPSE E200).

2.5. Hydrogen peroxide (H_2O_2) content

To evaluate the H_2O_2 content, the fresh cell pellet (0.1 g) was ground in a 2 mL solution of 0.1% (w/v) trichloroacetic acid at 4°C. After centrifugation (12,000 × g, 10 min at 4°C), the extract (500 µL) was added to a reaction mixture containing 0.5 mL potassium phosphate buffer (100 mM, pH 7.4) and 1 mL potassium iodide (1 M). The absorbance was read at 390 nm, and the H_2O_2 content was calculated using a standard curve [17].

2.6. Astaxanthin content

The fresh cell pellet (0.2 g) was added to the 5 mL KOH solution (5%) for astaxanthin evaluation. The mixture was incubated in a 70°C water bath for 5 minutes. After centrifugation (5,000 × g, 4 min), 5 mL of DMSO and 5 drops of acetic acid were added to the remaining cell debris, and the mixture was incubated in a 70°C water bath for 5 minutes [18]. After centrifugation, the absorbance was measured at 490 nm, and the astaxanthin content was calculated using the following formula.

$$c(\text{mg/L}) = 4.5 \times A492 \times Va/Vb$$

In this formula, Va and Vb are the DMSO volume and algal sample, respectively [19].

2.7. Total phenolic content

The fresh cell pellet (0.2 g) was ground in 3 mL of methanol to determine phenolic compounds and centrifuged at 7,000 × g for 10 min. Then, the 0.5 mL of extract was mixed with 0.5 mL Folin–Ciocalteu reagent 10% (Sigma-Aldrich) and vortexed. After 2 min, 3.2 mL sodium carbonate (5% w/v) was added to the reaction mixture. The absorbance was recorded at 740 nm after 30 min, and its content was evaluated by gallic acid (≥*99*% purity, Sigma-Aldrich) as a standard [20].

2.8. Statistical analysis

Statistical analysis was conducted using ANOVA in SPSS software (*Version 21*). T-test was conducted for growth analysis. Growth kinetics experiments were conducted in four replicates, and flight experiments were performed in three replicates for each treatment. Significant differences between means were evaluated by Duncan's test at the $P \leq 0.05$ level.

3. RESULTS

3.1. Kinetic growth curve of an alga

The kinetic growth of *H. pluvialis* cells was shown in Fig. 2. In the first 5 days, cells were in the lag phase. Then, cells entered the logarithmic growth phase until day 25 and followed the stationary phase (from day 26 until day 30), transforming from green aplanospores into red cyst cells. During the stationary phase, red pigments accumulated in cysts.

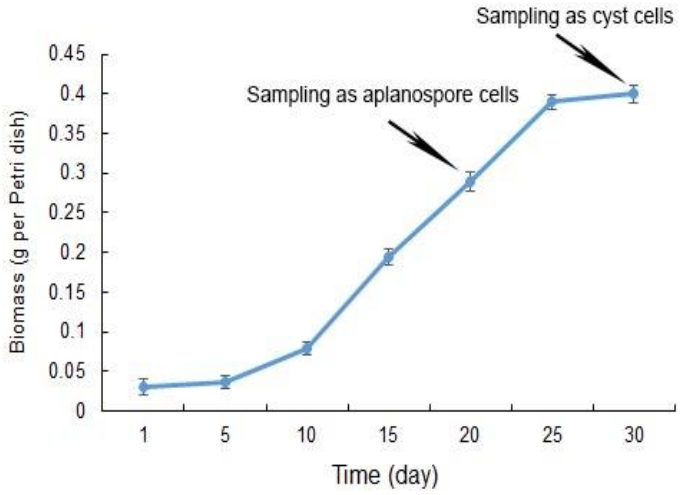

Fig. 2. The kinetic growth curve of *H. pluvialis* algal cells in the solid MS medium with sampling times for flight experiments.

3.2. Morphological study of cells after flight

In aplanospore samples, most cells (83% ± 2.93) were in the green aplanospore stage after two days post-flight in the control samples; However, a few numbers of cells (17% ± 1.67) were in spindle-shaped and motile (Fig. 3). In the flight-exposed samples, the number of motile cells did not change significantly compared to the control, but some aplanospore cells (35% ± 1.12) began to accumulate pigment.

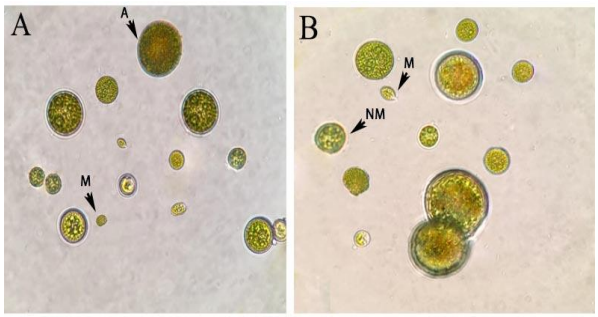

Fig. 3. Morphology of *H. pluvialis* cells from aplanospore stage after two days post-flight. **(**A) Control cells predominantly composed of green non-motile aplanospores and (B) flight-exposed samples show the initiation of pigment accumulation (reddening) in some aplanospore cells, indicating a stress response. M: motile cell, NM non-motile, A: aplanospore.

In the cyst samples, all cells were non-motile (100% ± 3.78) in the control samples. However, many cells were spindle-shaped (71% ± 2.69), red in color, and motile in the flight-exposed sample. The flight conditions lead to the release of red zoospores and cyst germination. Fig. 4B shows a red cyst and spindle-shaped zoospore in the flight-exposed sample. It also shows the germination of red cysts (GC).

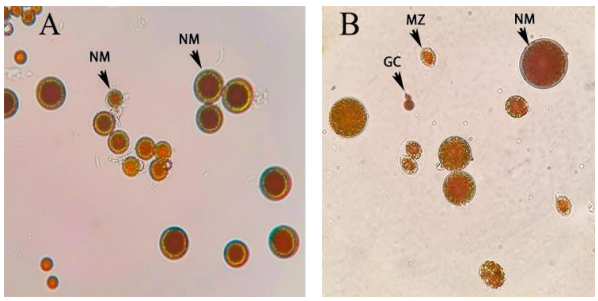

Fig. 4. Morphology of *H. pluvialis* cells from cyst stage after two days post-flight. (A) Cyst cells in the control conditions and (B) flight-exposed cells containing cyst and zoospore cells.

3.3. Cellular stage distribution

The analysis of cellular stage distribution after two days post-flight showed that the number of green aplanospore cells decreased significantly ($P \leq 0.05$) in the flight-exposed sample compared to the control, showing a 60.2% reduction. Meanwhile, some cells with anthocyanin accumulation (35%) were identified in the flight-exposed samples. On the other hand, in the cyst samples, the flight conditions induced a significant ($P \leq 0.05$) cyst germination, motile zoospores appeared, and their number reached to 71%. However, in the control sample, all cells were in the cyst stage (Fig. 5).

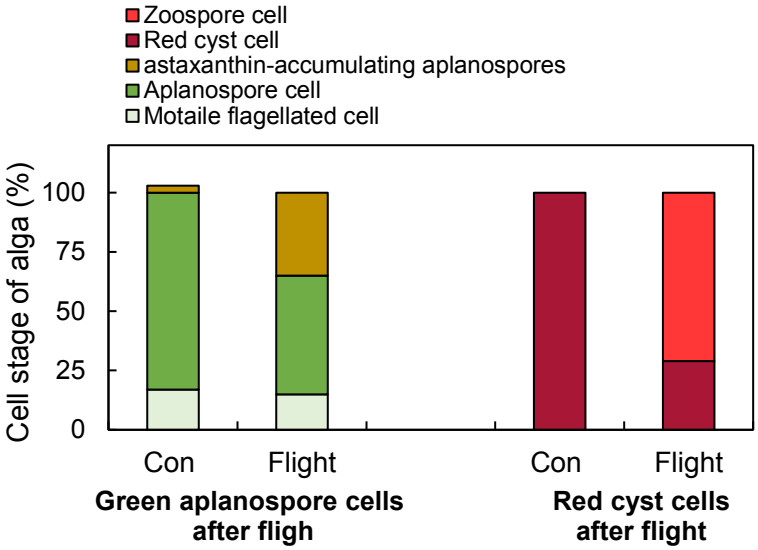

Fig. 5. Distribution of *H. pluvialis* cellular stages in the control and flight samples after 2 days of flight.

3.4. Impact of suborbital flight on the algal cell growth

The green aplanospore cells after flight are placed in culture room conditions for 7 days, and the obtained biomass is shown in Fig. 6. The growth results showed no significant difference in terms of growth between the control (0.42 g DW) and flight (0.38 g DW) cells.

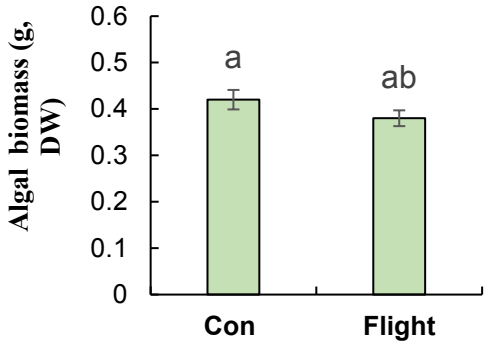

Fig. 6. Impact of suborbital flight on the *H. pluvialis* cell biomass after 7 days of flight. Different letters indicate significance at the $P \leq 0.05$ level.

3.5. Impact of suborbital flight on the H_2O_2 and astaxanthin contents

The flight conditions led to a significant change ($P \leq 0.05$) in H_2O_2 levels of aplanospore cells, particularly after 2 days (the logarithmic phase), resulting in a 2.1-fold increase compared to the control. After 7 days post-flight, cells entered the stationary growth phase, and H_2O_2 level showed a further rise in the control samples compared to 2 days. The aplanospore cells after 7 days post-flight exhibited a 1.3-fold increase in H_2O_2 content compared to the control (Fig. 7A). Moreover, the accumulation of astaxanthin in the algal cells showed a positive relation with H_2O_2 level. The highest astaxanthin content (26.1 mg g^{-1} FW) was observed during the stationary growth phase after 7 days post-flight by a 1.6-fold increase compared to the control (Fig. 7B).

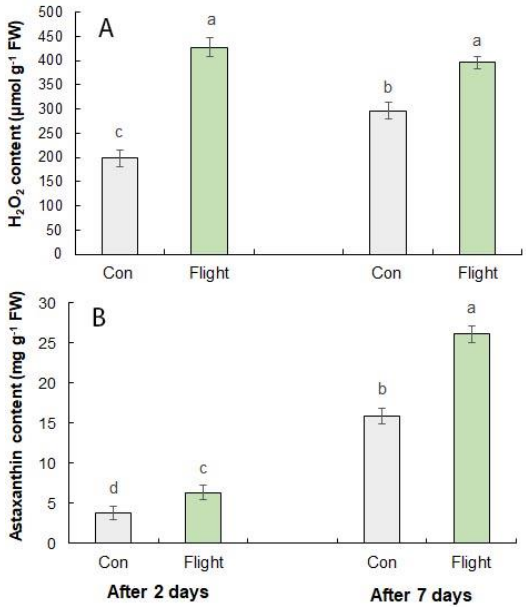

Fig. 7. Impact of suborbital flight on the H_2O_2 (A) and astaxanthin (B) contents of *H. pluvialis* after 2 and 7 days of post-flight. Different letters indicate significance at the $P \leq 0.05$ level.

3.6. Impact of suborbital flight on the total phenolics

Total phenolic content increased in flight-exposed cells comparing to the control. The highest phenolic content (935.6 mg g^{-1} FW) was observed after 7 days post-flight (at the stationary growth phase) in the flight-exposed samples, showing a 25.8% rise compared to the control. However, there was no significant difference in phenolic contents between the control and flight samples after 2 days post-flight

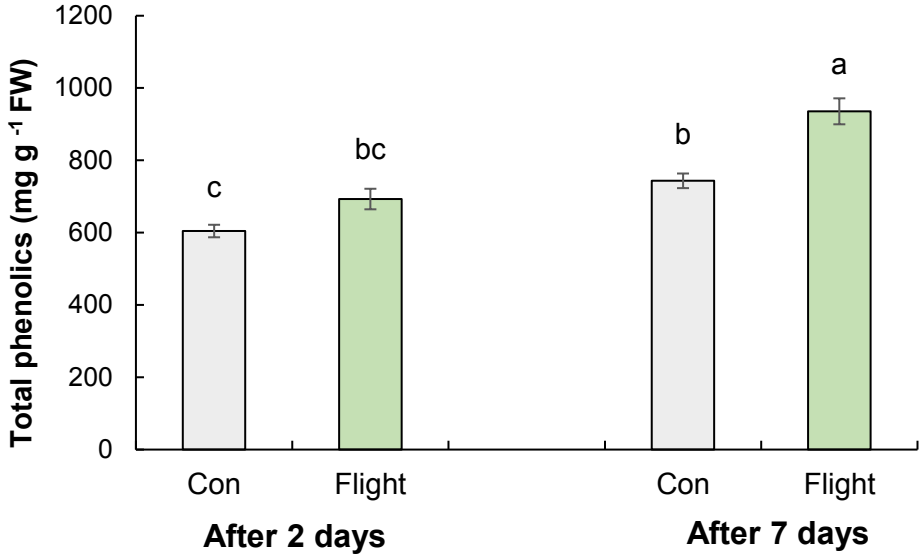

Fig. 8. Impact of suborbital flight on the total phenolics of *H. pluvialis* cells after 2 and 7 days of flight. Different letters indicate significance at the P ≥ 0.05 level.

4. DISCUSSION

Algae are promising candidates for oxygen production and antioxidant metabolites for long-duration space missions [1]. This study aimed to evaluate the feasibility of using suborbital environment as a trigger for enhancing the high-value antioxidant of astaxanthin and cyst germination to release zoospores. The algal growth kinetics showed three distinct phases on solid BBM medium: lag, logarithmic, and stationary. During the first 5 days, cells were in the lag phase and exhibited minimal growth. In this phase, cells were prepared to enter the logarithmic phase and began proliferation. Then, the cells entered the logarithmic phase until day 25, followed by the stationary phase (from day 26 to 30) (Fig. 1). During the stationary phase, algal cells transformed into cyst cells, and cellular defense mechanisms against oxidative damage were activated through the accumulation of antioxidant metabolites [16].

The study of cell morphology during suborbital flight revealed that cell responses vary across different growth phases. After two days of flight, the green aplanospores began accumulating the astaxanthin pigment, while the red cysts germinated and released zoospores (Figs 3 and 4). There is no data available on the impact of flight conditions on cyst germination; this is the first study. Cyst cells have a rigid cell wall, and some studies have shown that cyst germination can be stimulated under specific environmental and/or culture conditions. Bauer et al. (2021) reported that the heterotrophic (growth without light, using glucose or sodium acetate as a carbon source) and phototrophic conditions for the germination of *H. pluvialis cysts*. Heterotrophic conditions with glucose induced 80% of cysts to enter zoospore stage within 49 hours [10]. The findings of this study showed that suborbital flight could trigger metabolic processes to release zoospores, possibly by ROS regulation, which is in agreement with the results of H_2O_2 in this study. In suborbital experiments, biological samples are subjected to microgravity and low atmospheric pressure. Under low atmospheric pressure conditions, decreasing oxygen levels can activate ROS generation, and then the glycolysis cycle

induces to produce ATP [21]. During cyst germination, the glycolysis cycle and fatty acid oxidation are activated, preparing the initial energy and required precursors to initiate cellular metabolism and germination [22].

Flight conditions can influence cell growth and development. In this study, the flight conditions did not show a significant change in the cell growth, indicating that the algae cells could adapt to space conditions and could be considered a promising candidate for space research in the future. Various growth responses regarding the effects of flight conditions on algal growth have been reported. Similarly, Shevchenko et al. (1967) investigated the impact of flight conditions on *Chlorella* for 5 days in the crewed Vostok mission, and there was no significant change in cell growth between the spaceflight and control groups [23]. However, Wang et al. (2006) showed that *Anabaena* growth decreased under flight due to a temperature drop on the third and sixth days of the mission; but after the recovery period, the algal cultivation exhibited a higher growth rate than the control, although after several generations, both the flight and control cultures showed similar growth rates [15]. Qin et al. (2014) demonstrated that low atmospheric pressure (50 kPa) reduced the growth and biomass production in *Anabaena* microalgae, which was associated with a decline in chlorophyll content and an increasing carotenoid/chlorophyll ratio [24]. The effect of flight conditions on the algal growth responses seems to depend on the algal strain and flight conditions.

H_2O_2 plays an essential role in cellular signaling and is produced during stress by activating NADPH oxidases. Environmental stresses stimulate the production of various reactive oxygen species (ROS) in cells. These compounds can influence cell growth and metabolite production by activating signaling pathways related to the antioxidant defense system [25, 26]. In this study, a significant increase in H_2O_2 level was observed following suborbital flight, and the highest content was detected after 2 days of flight (the logarithmic growth phase). In *Arabidopsis*, suborbital launch of callus tissue for 20 seconds led to a rapid increase in cytoplasmic calcium and H_2O_2 levels. Increased calcium could activate calcium-dependent protein kinases for the phosphorylation of NADPH oxidases, and H_2O_2 production was triggered. Additionally, spaceflight could upregulate the expression of genes encoding antioxidant enzymes and metabolites [27]. On the other hand, findings of this study showed that the level of H_2O_2 after 2 days was higher than 7 days, indicating the regulation of ROS level by antioxidant metabolites at the stationary growth phase.

The regulation of antioxidant metabolism is closely related to the cell responses to oxidative stress. Signaling molecules affect gene expressions related to secondary metabolism by affecting protein kinases and MAPKs [28]. In this study, astaxanthin and phenolic contents increased with the rise in H_2O_2 level, and the highest accumulation of antioxidant metabolites was observed in the stationary phase after 7 days of flight. The accumulation of antioxidant metabolites can regulate the cellular ROS level. Zhao et al. (2019) reported that changes in medium composition increased the ROS level, astaxanthin, and fatty acid production in *H. pluvialis,* which was related to modulating oxidative stress [28]. Microarray analysis of *Arabidopsis* seedlings exposed to 10 min flight by an exploration rocket showed that the upregulation of 141 genes, which were related to the antioxidant defense system, metabolism, cell wall, and signal transduction [29]. Genes related to homoserine kinase (synthesis of amino acids), arginine decarboxylase (synthesis of polyamines), and aminotransferase (nitrogen fixation for protein biosynthesis) were also increased under space flight [29]. On the other hand, phenolic compounds are accumulated in cells to scavenge ROS and oxidative defense under stress conditions [30]. In this study, the total phenolic content reached 935.6 mg g^{-1} FW after 7 days, showing a 25.8% increase in the stationary growth phase compared to the control. Hassanpour and Abdel Latef (2022) demonstrated that microgravity increased phenolic and flavonoid compounds in *Ocimum bacilicum*, which was attributed to the activation of the PAL enzyme in the biosynthetic pathway [31]. The increase in antioxidant compounds led to the cessation of membrane damage and protection of cells under space conditions [32].

5. CONCLUSIONS

The cell responses of *H. pluvialis* to suborbital flight depend on the cell growth stage. In the aplanospore stage, cells after flight showed a tendency to accumulate astaxanthin pigments;

however, in the cyst stage, flight induced germination and the release of zoospores. The growth of algal cells did not change after flight, indicating that *Haematococcus* alga cells are resistant to flight conditions. Inducing astaxanthin and phenolic productions were associated with a significant rise in H_2O_2 level. The findings of this study showed that *H. pluvialis* is a promising candidate for future human-crewed missions, as food supplements can be produced in situ. More studies are needed to investigate the genes' response to astaxanthin production in the future.

6. ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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